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Mapping of the Dimer Interface of the *Escherichia coli* Mannitol Permease by Cysteine Cross-linking*

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A cysteine cross-linking approach was used to identify residues at the dimer interface of the *Escherichia coli* mannitol permease. This transport protein comprises two cytoplasmic domains and one membrane-embedded C domain per monomer, of which the latter provides the dimer contacts. A series of single-cysteine His-tagged C domains present in the native membrane were subjected to Cu(II)-(1,10-phenanthroline)$_3$-catalyzed disulfide formation or cysteine cross-linking with dimaleimides of different length. The engineered cysteines were at the borders of the predicted membrane-spanning α-helices. Two residues were found to be located in close proximity of each other and capable of forming a disulfide, while four other locations formed cross-links with the longer dimaleimides. Solubilization of the membranes did only influence the cross-linking behavior at one position (Cys^{73}). Mannitol binding only effected the cross-linking of a cysteine at the border of the third transmembrane helix (Cys^{134}), indicating that substrate binding does not lead to large rearrangements in the helix packing or to dissociation of the dimer. Upon mannitol binding, the Cys^{134} becomes more exposed but the residue is no longer capable of forming a stable disulfide in the dimeric IIC domain. In combination with the recently obtained projection structure of the IIC domain in two-dimensional crystals, a first proposal is made for α-helix packing in the mannitol permease.

In bacteria, the phosphoenolpyruvate-dependent phosphotransferase system is involved in the uptake and concomitant phosphorylation of a wide variety of carbohydrates (1). The system consists of two general components EI and HPr and several carbohydrate-specific enzymes II (EII). In a cascade of phosphorylation reactions, the phosphoryl group is transferred from the energy donor phosphoenolpyruvate via EI and HPr to EII, as is illustrated in Fig. 1 for the mannitol-specific phosphotransferase system (reviewed in Ref. 2). All EII's have a similar architecture and consist of the cytoplasmic A and B domains and a membrane-embedded C domain. In the mannitol-specific EII (EII^{mtl})[1] of *Escherichia coli* the three domains are covalently linked. The C domain transports mannitol into the cell and the A and B domains transfer the phosphoryl group from HPr to mannitol. The imported mannitol is phosphorylated by B while being bound by C.

The association state of EII^{mtl} is most likely the dimer with contacts between two subunits being provided by the C domain. The evidence for this oligomeric state is manifold, but the strongest indications are from *in vitro* and *in vivo* complementation studies. The formation of heterodimers from inactive subunits with defects in different domains restores the activity of EII^{mtl} (3–5). Moreover, subunits of EII^{mtl} could be covalently cross-linked to a dimer with bifunctional sulfhydryl-specific reagents, lysine-specific cross-linkers, or by oxidative disulfide bond formation (6–9). The 5-Å projection structure of the C domain in two-dimensional crystals also strongly points towards a dimeric structure (10).

Six regions with high electron density, probably representing α-helices, are observed in the projection structure of the C domain. The structural information of the C domain, however, is limited to this projection map. A topology model derived from the *phoA* gene fusion studies predicted 6 membrane-spanning α-helices and two large cytoplasmic loops (Fig. 2), which fits with the projection map (11). The assignment of densities to particular helices has not been made and, therefore, it is not known which helices reside at the dimer interface. Here, we demonstrate close proximity of engineered cysteines at the borders of predicted membrane-spanning α-helices, using Cu(II)-(1,10-phenathroline)$_3$-induced disulfide bridge formation and cross-linking with dimaleimides of different length. This data, in combination with the electron densities of the projection map, has been used to propose the first α-helix-packing model of a phosphotransferase system transport protein.

EXPERIMENTAL PROCEDURES

Chemicals—Decyl-polyethylene glycol (dPEG) was synthesized by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). 1,10-Phenanthroline monohydrate was obtained from Merck. The cross-linkers *o*-PDM and *p*-PDM were from Aldrich, and BMH was obtained from Pierce. *n*-Ethylmaleimide (NEM) was from Fluka and ^{14}C-NEM from NEN. All chemicals used were analytical grade.

Construction of Plasmids for Expression of the Single-cysteine Mutants—Site-directed mutagenesis was performed with the Stratagene QuikChange mutagenesis kit. A His-tagged Cys-less IIC domain (IIChis-CL) was generated by replacing Cys^{110} and Cys^{320} by serine in PmaMtlIICHis, which carries the gene for the C domain with a C-terminal His-tag (12). The gene for IIChis-CL was subsequently used to generate the single-cysteine IIChis variants. Mutations were confirmed by DNA sequence analysis.

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[1] The abbreviations used are: EII^{mtl}, mannitol-specific enzyme II; IIChis, His-tagged C domain of EII^{mtl}; IIChis-CL, Cys-less IIChis; IIChis-X#C, single-cysteine mutants in the IIChis-CL background; ISO, inside-out; CuPhe, Cu(II)-(1,10-phenanthroline)$_3$; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; dPEG, decyl-polyethylene glycol.

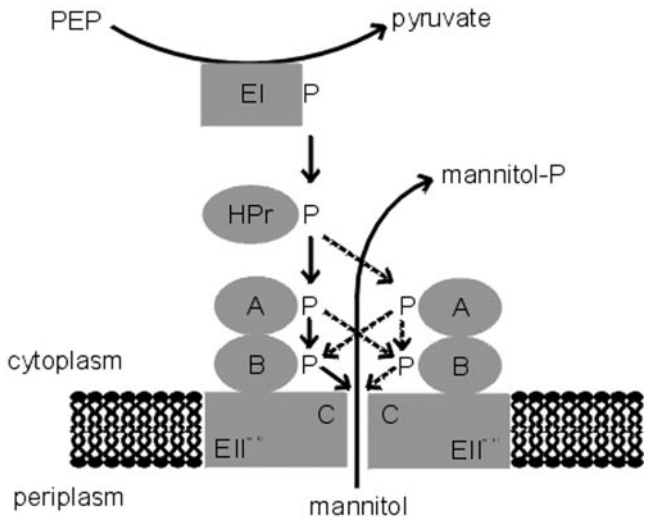

FIG. 1. **Schematic representation of the mannitol-specific phosphoenolpyruvate-dependent phosphotransferase system of *E. coli*.** *Dashed arrows* indicate that phosphoryl group transfer from HPr can proceed to each of the EII^{mtl} subunits and that inter- and intradomain phosphoryl group transfer is possible.

Isolation of ISO Membrane Vesicles and Membrane Solubilization—Growth of *E. coli* LGS322 (*F⁻ thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, gatR49, gatR50, Δ(mtlA'p), mtlDc, Δ(gutR'MDBA-recA)*), carrying the various plasmids, and procedures to overexpress the mutant proteins were identical to those described for wild type EII^{mtl} (13). Inside-out (ISO) membrane vesicles containing the mutant proteins were obtained as described (14). The membrane vesicles (25 mg of protein/ml) were solubilized in 1% (w/v) of dPEG. The protein concentration was determined with the DC protein assay from Bio-Rad.

Mannitol Binding and Phosphorylation Activities—The dissociation constants for mannitol binding and concentrations of the C domains were determined with flow dialysis in the presence or absence of 0.25% dPEG (w/v) (15) with some modifications as will be described elsewhere.[2] The functionality of the IIChis mutants was determined after heterodimer formation with EII^{mtl}-G196D, using 33 μM ^{14}C-mannitol following established procedures (16).

Generation of Heterodimers—Heterodimers between the single-cysteine IIChis and IIChis-CL (added at a 4-fold excess) were formed by mixing two preparations of dPEG (1%)-solubilized ISO membrane vesicles containing the separate IIC proteins. As a control a 4-fold excess of the appropriate single-cysteine IIChis mutant instead of IIChis-CL was added. To promote heterodimer formation, 167 mM Na_3PO_4 was added from a 1 M stock solution adjusted to pH 7.6 with HCl and these mixtures were incubated at 30 °C for 1 h. Na_3PO_4 lowers the cloudpoint of the dPEG, which results in dissociation of the initially homodimeric proteins and thereby facilitates the mixing of the species (17). To allow formation of the dimers, the Na_3PO_4 was removed by desalting of the dPEG-solubilized proteins on a Bio Microspin column (see the "Cysteine Cross-linking" procedure). Subsequently, 5 mM DTT was added and these mixtures were incubated for 15 min at 30 °C and treated further as described under the "Cysteine Cross-linking" procedure.

Cysteine Cross-linking—The ISO membrane vesicles or dPEG-solubilized proteins were desalted on a Bio Micro-spin 6 column (Bio-Rad), equilibrated in 25 mM Tris-HCl, pH 7.5. This buffer was deaerated with helium. The final protein concentration was ~5 mg/ml, from which 2–5% corresponds to IIChis. If appropriate, 0.01 or 40 mM mannitol or 40 mM glucose were added. Disulfide bridge formation was initiated by oxidation with 0.1 volume of 3 mM Cu(II)-(1,10-phenanthroline)$_3$ (CuPhe) and incubation at 30 °C for 0–60 min. The reaction was quenched by the addition of 20 mM EDTA plus 5 mM NEM. Cross-linking with dimaleimides of varying length was initiated by adding 0.05–0.5 mM *o*-PDM, *p*-PDM, or BMH from 10 times concentrated stock solutions in *N,N*-dimethylformamide. The reaction was stopped with 5 mM DTT after incubation at 30 °C for 0–60 min.

To generate cross-links between subunits with a single cysteine residue at different positions, heterodimers were formed as described under "Generation of Heterodimers" after mixing of two single Cys mutants in a one to one or one to four ratio. A one to four ratio was used when one of the mutants already formed a cross-link in the homodimeric situation. In that case, the mutant with the selfcross-link was used at a 4-fold lower concentration. The mutants that did not form a selfcross-link were mixed in a one to one ratio.

Accessibility of Cysteines—The ISO membrane vesicles containing the His-tagged C domains were treated with *p*-PDM for 0 or 60 min under the same conditions as described under "Cysteine Cross-linking," but the reaction was quenched with 10 mM β-mercaptoethanol. Subsequently, the His-tagged C domains were purified as described (12). Unreacted cysteines were kept reduced with 5 mM DTT, and the reductant was removed with a Micro-spin 6 column (Bio-Rad) just prior to labeling with 100 μM ^{14}C-NEM for 0–30 min at room temperature. The reaction was quenched by the addition of SDS-PAGE denaturation buffer with β-mercaptoethanol. The samples were analyzed with SDS-PAGE and autoradiograms were made with a PhosphorImager (Molecular Dynamics) after drying of the gel. The amount of radioactivity in the protein bands was determined with the Imagequant Software (Molecular Dynamics).

The Effect of Modification of Cys^{134} on the Phosphorylation Activity—ISO membrane vesicles with IIChis-S134C were labeled with 0.5 mM NEM or BMH under the same conditions as described under "Cysteine Cross-linking" in the presence or absence of 40 mM mannitol for different periods of time. The reaction was quenched with DTT. The reagents were removed by three consecutive washes with 25 mM Tris-HCl, pH 7.5, using ultracentrifugation at 200,000 × *g* for 10 min, and resuspension of the pelleted membranes. The activities of the proteins were determined using established procedures (16) as described under "Mannitol Binding and Phosphorylation Activities" with the following modifications. To 50 μg of ISO membrane vesicles (corresponding to ~70 nM IIChis-S134C), 10 μM of purified IIAB domain, 9 μM HPr, and 0.3 μM EI were added. The assay was started by the addition of 33 μM [^{14}C]mannitol; dPEG was omitted from the mixture.

SDS-PAGE Analysis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was done with 10% acrylamide gels as described (18). A denaturation buffer without β-mercaptoethanol was used. The proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using the Western-Light™ chemiluminescence detection kit with CSPD™ as the substrate, was performed as recommended by the manufacturer (Tropix Inc.). The first antibody was an anti-His antibody from Amersham Bioscience or Roche Molecular Biochemicals, and the second antibody was an anti-mouse IgG alkaline phosphatase conjugate (Sigma).

RESULTS

Generation and Characterization of the Mutants—Cysteines were introduced in a cysteine-less His-tagged C domain, denoted here as IIChis-CL, to identify putative transmembrane α-helices that constitute the dimer interface of EII^{mtl}. Fig. 2 shows the location of the cysteines in the topology model of the C domain. The cysteines, except for position 124, are located at the borders of putative transmembrane α-helices and they are taken as indicators for interhelix proximities.

ISO membrane vesicles prepared from LGS322 cells harboring the plasmids were analyzed for mannitol binding before and after solubilization with dPEG. Their ability to complement the mannitol binding-defective EII^{mtl}-G196D was determined in an *in vitro* mannitol phosphorylation assay (16). Table I shows the dissociation constants (K_d) for mannitol. Most proteins exhibited binding affinities comparable with those described for the wild-type C domain generated by tryptic digestion of the complete protein (15, 19); the maximal number of binding sites (B_{max}) did not vary significantly for the various mutants (not shown). A few single-cysteine mutants displayed significantly higher affinities than IIChis. The only mutant that bound mannitol with lower affinity was S134C. Although its binding constant could not be determined accurately with flow dialysis, it was estimated to be in the low micromolar range. All mutants, including S134C, complemented EII^{mtl}-G196D in the *in vitro* phosphorylation assay (not shown) with an efficiency comparable with that described for the C domain

[2] E. Vos, G. K. Schuurman-Wolters, G. T. Robillard, L. Broos, and B. Poolman, unpublished data.

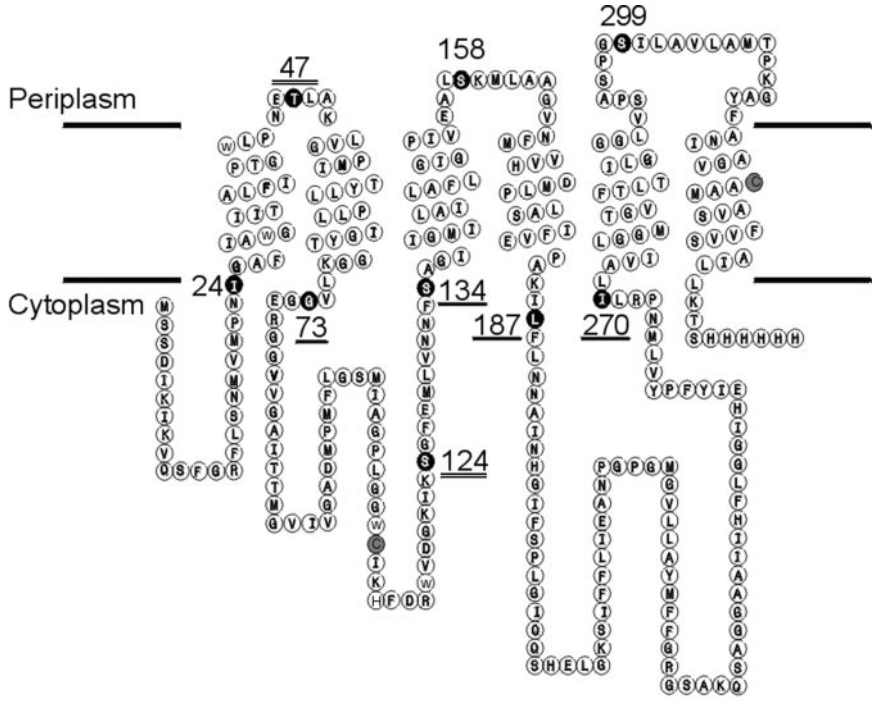

FIG. 2. **The location of the cysteines in the Sugiyama membrane topology model of the C domain of EII^{mtl}.** The positions of the two native cysteines, Cys^{110} and Cys^{320}, which were replaced with a serine, are shown in *gray*. The locations of the residues that were replaced with a cysteine are shown in *black*. *Double-underlined* residues do form a cross-link with CuPhe, while *single-underlined* residues form cross-links with the longer dimaleimides.

TABLE I
Dissociation constants for mannitol binding in the absence and presence of 0.25% dPEG

Mutant	K_d	K_d +dPEG
	μM	
IIChis	240	106
IIChis-CL	118	32
IIChis-I24C	55	18
IIChis-T47C	103	51
IIChis-G73C	116	50
IIChis-S124C	165	72
IIChis-S134C	>500[a]	97
IIChis-S158C	90	18
IIChis-L187C	124	62
IIChis-I270C	110	42
IIChis-S299C	169	54

[a] The binding constant could not be determined accurately.

(19). These data indicate that all IIChis mutants have more or less wild-type properties.

Cysteine Cross-linking of the Single-cysteine C Domains in Homodimeric Complexes—A disulfide bond is a so-called zero-length cross-linker, whereas the dimaleimides *o*-PDM, *p*-PDM, and BMH allow linkage of thiol groups at distances of 9.4 ± 0.5, 11.1 ± 0.5, and 10.2 ± 2.4 Å, respectively (20). Since an aromatic ring couples the maleimides in *o*-PDM and *p*-PDM, the span widths do not vary too much in solution. With BMH, on the other hand, the span width can vary a few Å, due to cis-trans conversions of the spacer chain. Representative immunoblots obtained with IIChis-T47C, IIChis-G73C, IIChis-S134C, and IIChis-S299C are shown in Fig. 3*A*. Table II summarizes the results of all mutants. Two mutants, with cysteines at positions 47 or 124, were able to form a disulfide bond and cross-link with all three dimaleimides. This indicates a high degree of flexibility for these cysteine residues since disulfide formation necessitates close proximity, whereas BMH and the rigid dimaleimides *o*-PDM and *p*-PDM span a larger distance. The capacity to form a disulfide indicates that these residues can come in very close proximity, since the length of a disulfide bond is ~2 Å (21, 22). Cross-linking with the cysteine at position 124 has been observed previously with native EII^{mtl} and purified IIChis-S124C (9). Four other mutants, with cysteines at positions 73, 134, 187, or 270, could only form a cross-link with *p*-PDM and BMH. This indicates that these locations are in close proximity but more than 10 Å apart. From the reaction with *p*-PDM and BMH, it can be concluded that the distance is ~12 Å. The cross-link with IIChis-G73C was formed less rapidly than those with cysteines at the other positions and significant amounts of cross-link product were only observed when the dimaleimide concentration was increased from 0.05 to 0.5 mM. This suggests a lower accessibility of the cysteine at position 73 or a higher pK_a of the sulfhydryl group. IIChis, with the native cysteines 110 and 320, and IIChis with cysteines at positions 24, 158, and 199 did not yield cross-links, suggesting that these cysteines are not in close proximity of the dimer interface or not accessible for the cross-linker.

The accessibility of all single-cysteine IIC mutants was examined by labeling with ^{14}C-NEM after pretreatment with *p*-PDM for 0 up to 60 min. The experiments showed that *p*-PDM protected against labeling with radioactive NEM in all cases (results not shown), demonstrating that each of the cysteines was accessible under the conditions used here. A slightly lower rate of labeling with ^{14}C-NEM was observed with IIChis-G73C and IIChis-I270C, which supports the need for higher concentrations of dimaleimide to obtain cross-links with the G73C mutant. Except for Cys^{134} (see below), mannitol binding did not alter the accessibility of the cysteines for the maleimides.

The effect of dPEG on the cross-linking efficiency was examined for the homodimeric single-cysteine C domains. In most cases, the data were comparable to those reported for the membrane-embedded protein (Fig. 3*A* and Table I). Detergent-solubilized G73C, on the other hand, formed stable cross-links in the presence of Cu-Phe and *p*-PDM (Fig. 3*B*), whereas this mutant only (slowly) formed cross-links with *p*-PDM (and BMH) when the protein was embedded in the membrane. Most likely, an increased flexibility in the presence of detergent allows the Cys^{73} residues to come closer together and form a stable disulfide.

Cysteine Cross-linking of the Single-cysteine C Domains in Heterodimeric Complexes—Due to the symmetry of the puta-

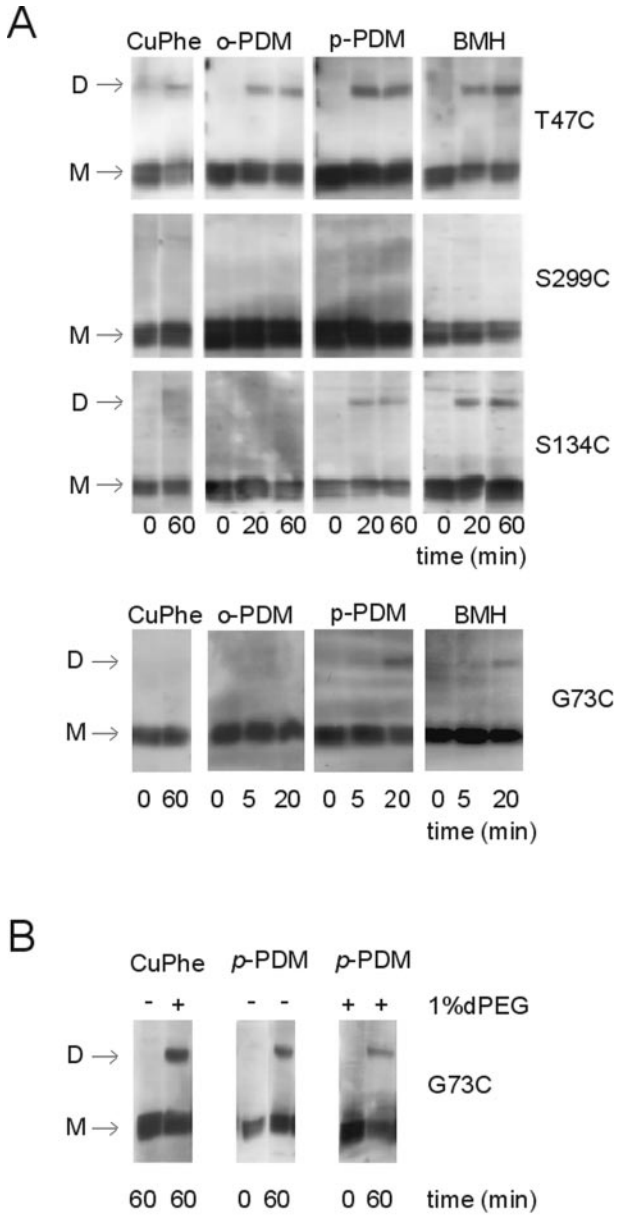

FIG. 3. **Immunoblots of cross-linking experiments.** *A,* immunoblots of cross-links observed with T47C, G73C, S299C, and S134C. Cross-links were formed by oxidation with 0.3 mM CuPhe or reaction with 0.5 mM *o*-PDM, *p*-PDM, or BMH, using intact ISO membrane vesicles. *M* refers to the monomer and *D* to the dimer. *B,* the effect of detergent on the cross-linking efficiency. IIChis-G73C was cross-linked via oxidation with 0.3 mM CuPhe or reaction with 0.5 mM *p*-PDM after solubilization of the membranes in the presence of 1% (w/v) dPEG. The reactions were allowed to proceed for the indicated periods of time before they were quenched with EDTA plus NEM (CuPhe oxidation) or DTT (dimaleimide cross-linking).

tive IIC dimer, only one helix is expected to be adjacent to the same helix in the other subunit. To increase the possibilities for stable cross-link formation, heterodimeric complexes of the following pairs of single-cysteine IIChis domains were formed: T47C/S158C, T47C/S299C, S158C/S299C, I24C/G73C, I24C/L187C, I24C/I270C, G73C/L187C, and G73C/I270C. The generation of heterodimers was accomplished by mixing dPEG-solubilized ISO membrane vesicles of given mutant pairs in the presence of 167 mM Na_3PO_4. The addition of sodium phosphate facilitates the heterodimer formation by lowering the cloud point of the detergent (see "Experimental Procedures"). Provided that homo- and heterodimeric complexes are formed randomly upon mixing of two species (A and B) in a one to one ratio, one expects 50% of heterodimers (AB) and 25% of each of the homodimers (AA and BB). A one to one ratio was used for those mutants that did not form a cross-link in the homodimeric complexes. A one to four ratio was used when one of the mutants (A), *i.e.* T47C, G73C, L187C, and I270C, already formed a selfcross-link. In that case, the other mutant (B) was added at a 4-fold excess and the expected fractions of AA, AB, and BB are 0.04, 0.32, and 0.64, respectively. Under these conditions the fraction of protein (AA) that could form a self-cross-link is only 4%. Cross-linking was tested with CuPhe and *p*-PDM but neither of the heterodimeric complexes formed significant amounts of cross-links. In some cases (S158C/S299C, I24C/G73C), the fraction of monomeric C domain decreased significantly after cross-linking, but an accompanying increase in dimeric species was not observed. Instead, these mutants seemed to aggregate in the presence of detergent plus Na_3PO_4 to promote heterodimer formation.

Intradimeric Cross-links Are Formed—From the previous experiments, it was not possible to discriminate between intra- or interdimeric cross-links. The latter would indicate that the particular cysteine is located at the periphery of the protein and not relevant for dimer formation. To discriminate between these possibilities, heterodimers composed of one single-cysteine and one cysteine-less subunit were formed. An intradimer cross-link is then no longer possible. The generation of heterodimers was accomplished by mixing dPEG-solubilized ISO membrane vesicles, containing one of the six single-cysteine C domains that did form a cross-link, with a 4-fold excess of solubilized membranes containing IIChis-CL in the presence of 167 mM Na_3PO_4. As a control a 4-fold excess of the same single-cysteine mutant was used instead of IIChis-CL. Fig. 4 shows that dPEG solubilization and lowering of the cloud point did not affect the cross-linking efficiency at positions 47, 73, 187, and 270 (*lanes 2* and *3*). When present in a heterodimer with IIChis-CL, these cysteines no longer form significant amounts of cross-links (*lane 4*). In the control sample, the cross-linked dimer is still clearly visible (*lane 5*). This indicates that cysteines 47, 73, 187, and 270 are located at the dimer interface. The two other single-cysteine mutants, with cysteines at position 124 and 134, could not be analyzed with this procedure, since Na_3PO_4 treatment of the dPEG-solubilized proteins resulted in aggregation. The cross-linking behavior of the S124C mutant has previously been analyzed in EII^{mtl} with cysteines at position 384 and 124 (9). When a heterodimer of this double-cysteine protein with IIChis-CL was formed, the Cys^{124}-Cys^{124} disulfide was no longer observed. This indicates that also the cross-link formed with IIChis-S124C is intradimeric.

Mannitol-induced Conformational Changes—The cross-linking experiments were repeated in the presence of 0.01 or 40 mM mannitol; both concentrations are above the K_d for mannitol binding. As a control, 40 mM glucose was used, which is not a substrate of EII^{mtl}. Representative immunoblots of the effect of mannitol on the formation of cross-links with 0.5 mM BMH are shown in Fig. 5. The addition of mannitol or glucose to IIChis-T47C did not have an effect on the cross-linking efficiency. With IIChis-S134C, on the other hand, cross-links were no longer observed in the presence of 0.01 and 40 mM mannitol, while glucose had no effect. Similar results were obtained with *p*-PDM as the cross-linker. The cross-linking behavior of the other mutants was not affected (not shown). If high concentrations of substrate would monomerize the dimer, as suggested previously on the basis of SDS extractions of the protein from the membrane in the presence of 40 mM mannitol (23, 24), the amount of cross-link product should also have decreased for Cys^{47}, Cys^{73}, Cys^{124}, Cys^{187}, and Cys^{270}. Since this is not the

TABLE II
Cysteine cross-linking of single-cysteine mutants of IIChis with CuPhe or dimaleimides of different length

Mutant	CuPhe	*o*-PDM (9.4 ± 0.5 Å)[a]	*p*-PDM (11.1 ± 0.5 Å)[a]	BMH (11.2 ± 2.4 Å)[a]
IIChis	−	−	−	−
IIChis CL	−	−	−	−
IIChis-I24C	−	−	−	−
IIChis-T47C	+	+	+	+
IIChis-G73C[b]	−/+[c]	−	+	+
IIChis-S124C	+	+	+	+
IIChis-S134C	−	−	+	+
IIChis-S158C	−	−	−	−
IIChis-L187C	−	−	+	+/−[d]
IIChis-I270C	−	−	+	+/−[d]
IIChis-S299C	−	−	−	−

[a] The span widths are taken from Ref. 20.
[b] These cross-links were formed with 0.5 mM dimaleimide and were only visible after 60 min of incubation.
[c] The disulfide was only observed after dPEG solubilization of the membranes.
[d] Unambiguous cross-link formation with BMH could not be obtained for the cysteine at this position.

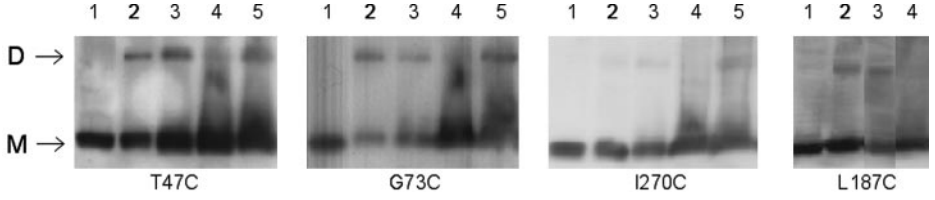

FIG. 4. **Heterodimer experiments with IIChis-T47C, IIChis-G73C, and IIChis-I270C to determine whether the cross-links are intra- or interdimeric.** Cross-linking with 0.5 mM p-PDM in 1% (w/v) dPEG-solubilized ISO membrane vesicles. *Lanes 1*, no cross-linking. *Lanes 2*, cross-linking in ISO membrane vesicles. *Lanes 3*, cross-linking in detergent-solubilized membrane vesicles after Na_3PO_4 treatment. *Lanes 4*, cross-linking in detergent-solubilized membrane vesicles with a 4-fold excess of IIChis-CL. *Lanes 5*, cross-linking in detergent-solubilized membrane vesicles, with 4-fold excess of the same mutant. The reactions were allowed to proceed for the indicated periods of time before they were quenched with EDTA plus NEM (CuPhe oxidation) or DTT (dimaleimide cross-linking).

case, it can be concluded that mannitol does not monomerize the dimer under the conditions used here.

The effect of labeling of Cys^{134} on the activity of the protein was investigated by complementation of IIChis-S134C in ISO membrane vesicles with purified IIAB protein of EII^{mtl}. Upon phosphorylation of IIAB in the presence of phosphoenolpyruvate, enzyme, and HPr, the phosphoryl group on the B domain of IIAB can be transferred to mannitol when bound to IIChis-S134C. In the absence of mannitol, NEM or BMH treatment (0.5 mM) resulted in about 25% reduction of the activity after 20 min of incubation (Fig. 6). Surprisingly, in the presence of mannitol, the inhibition by NEM or BMH was much more severe (Fig. 6) and already maximal after 5 min (not shown). The binding of mannitol thus results in a higher accessibility (or reactivity) of Cys^{134}, but also places these residues further apart as cross-linking is no longer observed under these conditions (Fig. 5).

DISCUSSION

The cross-linking experiments with the C domain of the mannitol permease have been primarily performed with the protein in ISO membrane vesicles, which is the environment in which the protein adopts its native conformation. The lipid bilayer environment has the additional advantage that protein motions are restricted. This could be important as cross-links can also be formed upon dynamic collisions, which would not merely reflect proximity of the subunits. With a membrane-embedded protein, the collisions can only occur within the two-dimensional space of the membrane, which increases the specificity of the cross-linking reactions, as was shown for the diacylglycerol kinase (25). A CuPhe-catalyzed disulfide formation study showed that collision rates are highly dependent on proximity (22). A comparison of cysteine cross-link formation and distance determination by spin-spin interactions between nitroxide-labeled pairs of cysteines in the *E. coli* lactose per-

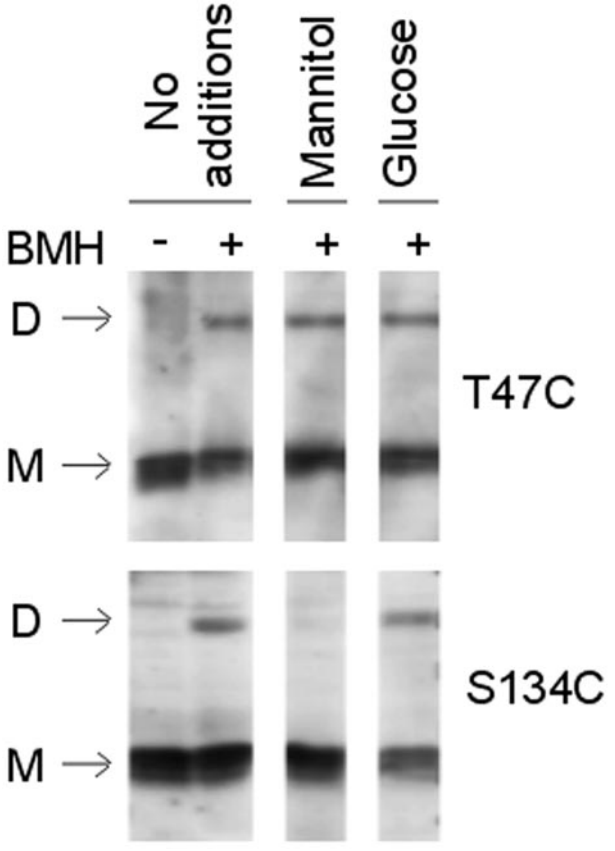

FIG. 5. **Effect of mannitol on cross-linking.** IIChis-T47C and IIChis-S134C were cross-linked with 0.5 mM BMH without further additions or in the presence of 40 mM mannitol or glucose. ISO membrane vesicles were used and the reaction was quenched with DTT.

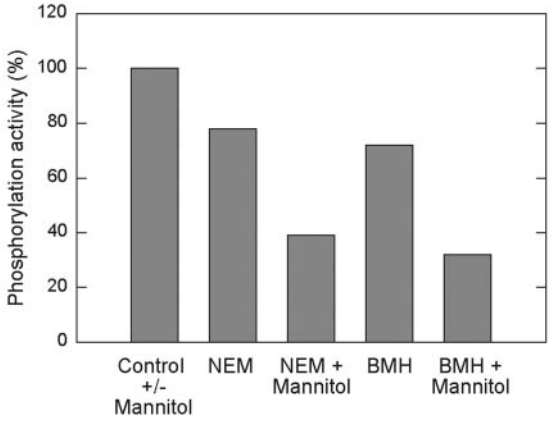

FIG. 6. **Effect of modification of Cys^{134} on the phosphorylation activity.** ISO membrane vesicles with IIChis-S134C were labeled with NEM or BMH in the presence or absence of 40 mM mannitol for different periods of time and the residual activity was determined. Shown here is the residual activity after 20 min.

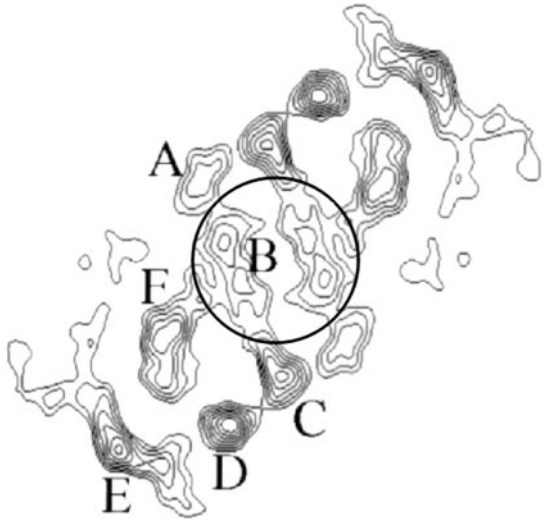

FIG. 7. **The projection map of the C domain of EII^{mtl}.** The projection map was taken from Koning *et al.* (10). The *drawn circle* has a diameter of 25 Å. The different densities are denoted in *capital letters*.

mease also indicates that proximity is the most important parameter (26). This fits with our observations that the cross-link efficiency of mutants is similar for most of the single-cysteine mutants in the native and dPEG-solubilized membrane. For the G73C mutant, however, dPEG solubilization is required for disulfide formation, whereas cross-linking via the dimaleimides is unaffected. This suggests that the two cysteines are brought in somewhat closer proximity upon solubilization of the membrane.

The relatively low efficiencies of CuPhe-induced disulfide bond formation or dimaleimide cross-linking are not uncommon (26–28) and there are several explanations for these observations. (i) The formation of disulfide bonds under oxidative conditions is competitive with terminal oxidation of thiols to sulfonates (22). (ii) A study by Koshland and co-workers (29) showed that the inefficiency of CuPhe-catalyzed cross-linking can relate to a low stability of the disulfide bond, which is due to the less than ideal geometries and bond lengths of the engineered cysteines. Even disulfide bonds between cysteines, engineered on the basis of a high-resolution crystal structure, can be difficult to form and unstable once formed (30). (iii) Cross-linking of two cysteines with one dimaleimide is competitive with the labeling of the same two cysteines with two different dimaleimide molecules, which obviously does not lead to cross-linking. In addition, cross-linking will not be observed if one of the maleimide moieties is inactivated by hydrolysis.

The observed cross-links can be divided into two groups. The first group is composed of residues 47 and 124, which form a disulfide bond upon CuPhe-induced oxidation and cross-links with all three dimaleimides. Since hydrophaty predictions and *phoA* gene fusion data suggest that residue 124 is not located at the border of a membrane-spanning α-helix, it is not expected to place a particular helix at the dimer interface. Residue 47 is in a loop between helix 1 and 2 and probably located in the center of the projection map close to density B (see Fig. 7). Residues 73, 134, 187, and 270 are in the second group of cross-linking sites. These cysteines, which are on the cytoplasmic side of helices 2, 3, 4, and 5, respectively, only form cross-links with the reagents with the longer spacers in the native membranes. The cytoplasmic ends of these helices must, therefore, be reasonably close to the dimer interface. Due to the instability of IIChis-S134C in the dPEG-solubilized state in the presence of sodium phosphate, we cannot exclude the possibility that residue 134 forms an interdimeric cross-link. Given the high specificity of cross-link formation with the other cysteine pairs, we favor the suggestion that the Cys^{134} cross-link is also intradimeric and sensitive to mannitol binding. The observation that mannitol enhances the inactivation of IIChis-S134C by NEM and BMH, whereas the cross-linking by BMH is diminished, indicate that relatively large conformational changes take place at this site upon ligand binding. The Cys^{134} residues become more exposed, and react more rapidly with the maleimides. At the same time, the conformational changes places the two Cys^{134} residues within the homodimer further apart, as BMH-dependent cross-linking is no longer observed.

With regard to the distance information obtained from the cross-linking studies, it is important to note that the distance between the centers of two densities in the projection map might be larger than the span width of the dimaleimides. The radius of a helix is 5–6 Å and ~12 Å can thus be added to the span width of the cross-linker used. The maximum distance between the centers of two densities that can be spanned by BMH is, therefore, ~25 Å. In Fig. 7, a circle with this diameter is drawn in the center of the projection map. Since density E is far out of the circle, it can never represent helices 1, 2, or 5 and most likely also not helix 3. The fact that residues 47 and 73 at either end of helix 2 form a cross-link makes this helix the best candidate for density B. This leaves densities A, C, F, and probably D for helices 1, 3, 4, and 5. Due to the symmetry of the putative IIC dimer different helices of the two subunits could neighbor each other. Helix 1 of one subunit could, for instance, be adjacent to helix 3, 4, or 5 of the other subunit. Unfortunately, neither of the mutant pairs in the heterodimeric complexes yielded significant amounts of cross-links to support these possibilities.

Although integral membrane proteins do not readily form three-dimensional crystals, two-dimensional crystals, from which a projection map can be calculated, are much more easily obtained. In fact, two-dimensional crystals of several integral membrane proteins have been produced in recent years (31–34). These maps reveal the electron densities corresponding to transmembrane α-helices, but assignment of the densities to particular segments of the protein cannot be made. Such information is particularly important for oligomeric proteins, in which the subunit interface(s) are catalytically important or participate in the cooperativity of the system (35). The approach presented here allows one to generate a helix-packing model of an integral membrane protein. Specific cross-links could be generated between cysteines engineered at the ends of the transmembrane segments of the membrane domain of

EII^{mtl}. In combination with the projection map of the C domain of EII^{mtl}, we have obtained the first structural information of the dimer interface. To the best of our knowledge, the two pieces of information, *i.e.* the electron density map and cross-links, have not been used before to build an α-helix packing model of a membrane (transport) protein.

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